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Original

In Vivo Tissue Response of Endodontic Bio-ceramic Materials

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Abstract: In order to evaluate the biocompatibility and mineral repair capacity, mineral trioxide aggregate (MTA), portland cement normal setting (PCn) and fast setting (PCf) and calcium hydroxide-based paste (Calen) filled silicone tube were implanted into the dorsal subcutaneous connective tissues of 25 *Wistar* rats. Animals were euthanized at 24, 72 hours, 7, 15 and 30 days. Implants with surrounding tissues were fixed with 10% buffer formaldehyde and processed for histological routine techniques. Slides (5 μm serial cuts) were stained with H&E and Von Kossa stains for morphological, qualitative and quantitative analysis by light microscopy. Calen showed severe and moderate inflammatory response and granulomatous reaction with psammoma body-like formation. PCn and MTA have similar behavior, with mild inflammatory reaction from 8% and 4%, respectively. Even though, PCn and MTA demonstrated analogous biological reaction, MTA developing thick artificial mineral precipitation (p = 0,007). All sealers demonstrated a similar inflammatory response at all time periods studied (p = 0.678).

Key words: Biocompatibility, Calcium hydroxide, Mineral trioxide aggregate, Portland cement.

Introduction

In the last thirty years, bio-ceramic materials were introduced in Medical and Dental fields. They are classified as I-Bioinert: non interactive with biological systems (Alumina, Zirconia), II-Bioactive: when produce interfacial interactions with surrounding tissue (Bioactive glasses, Bioactive glass ceramics, Hydroxyapatite, Calcium silicates) and III-Biodegradable: soluble or resorbable, eventually replaced or incorporated into tissue (Tricalcium phosphate, Bioactive glass[1)]). Bio-ceramics have ability to either functions as natural tissues or to be resorbed and stimulate natural regeneration[1,2)]. Recently, bio-ceramic based sealers were introduced in endodontic, categorized as I Calcium silicate based-cements with mineral trioxide aggregate (MTA) and portland cement; II Calcium phosphate, Tricalcium phosphate, Hydroxyapatite, Calcium Hydroxide based sealers. And III Mixture of Calcium Silicates and Calcium Phosphates based sealers[2,3)]. Portland Cement used in the construction industry as a binder in concrete, lately have been introduced in dentistry. The first-generation material was mineral trioxide aggregate (MTA) composed of Portland cement and bismuth oxide[4)]. The Portland cement component when mixed with water results in the formation of calcium silicate hydrate, calcium hydroxide and ettringite[5)]. To detect chemical elements, samples of MTA and Portland cement were analyzed by inductively coupled plasma emission spectrometry (ICP-ES)[6)]. Comparative analysis for fifteen different elements revealed a significant similarity between them, with the exception that there was no detectable quantity of bismuth in Portland cement[7)]. There are many studies suggesting that MTA is a suitable material to be used as retrograde filling cement, perforations and pulpotomies, due to its good sealing ability, biocompatibility and deposit of hard tissue at dental apices and dental pulp [8-12)]. Related to PC, it was demonstrated its similarity to MTA[5-7,13)], it has high alkalinity (pH 10), which inhibits bacterial proliferation[14)], good sealing and some studies consider PC a substitute for MTA[15)]. However, there is scarce research concerning the biological tissue response from Portland Cement materials *in vivo*[15,16)]. From Group II called Calcium phosphate-based sealers, Calcium Hydroxide was selected for its ability to react with the surrounding tissues. The Calcium hydroxide Ca (OH_2) is widely used based on its biological, physic-chemical, antimicrobial, among other characteristics[17)].$CaOH_2$ materials have been used for root canal sealers, retrograde root fillings, intra-canal medicament between appointments, apexogenesis, arrest of root resorption defects, and direct or indirect pulp-capping procedures[18)]. Although calcium hydroxide has been widely accepted in clinical practice, some studies show it may be cytotoxic due to high alkalinity[19,20)] This characteristic is due to the release of hydroxyl ions responsible for antimicrobial actions, and for promoting mineralized tissue formation. Our research was focalized to evaluate by ISO international standards (10993-6 and 7405)[21,22)], the local effects from Portland Cement (normal and fast

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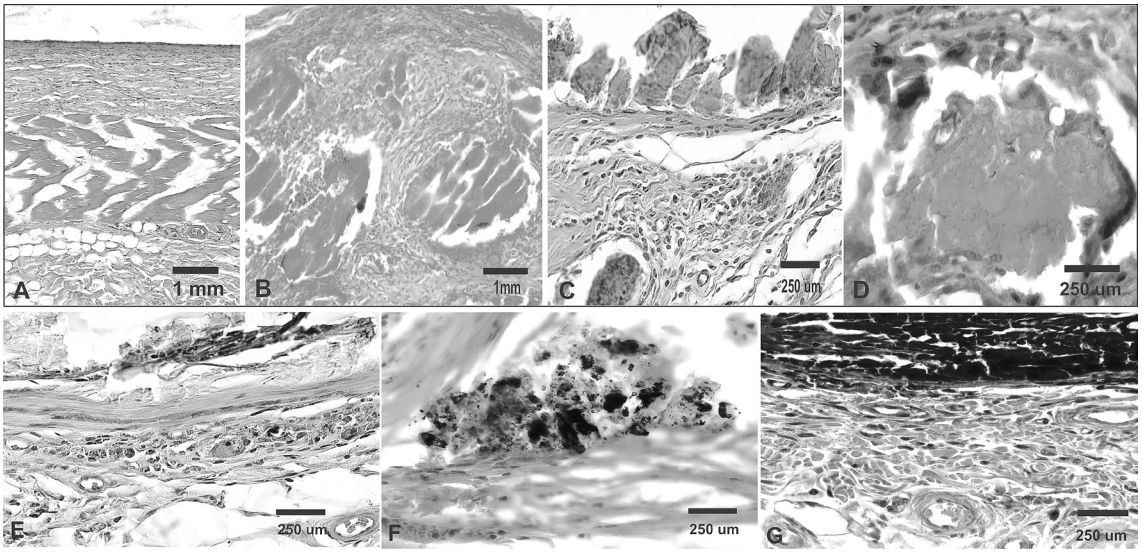

Figure 1. A: Negative control group at 30 days. Numerous fibroblasts, fibrocytes as well as fibrous, muscle and adipose tissues were observed. No inflammatory cells were found. H&E stained. Magnification 100 X, bar = 1 mm. B: Calen at 30 days. Inflammatory granulomatous tissue reaction surrounding crystallized structures called psammomas body like formation, with giant foreign body cells distributed around them were found. H&E staining. Magnification 100X, bar = 1mm. C: Calen at 30 days. Numerous amorphous laminar structures deposited at the tube end surrounded by giant cells were found. H&E stained. Magnification 400 X, bar = 250 μm. D: Calen at 30 days. Dystrophic calcified structure like psammoma body were observed, at high magnification. H&E stained. Magnification 600 X, bar = 250 μm. E: PC normal setting at 30 days. Presence of dark granular crystal precipitation. Fibrous-hyalinized-like capsule. Macrophages engulfing foreign material and congestion were observed. Magnification 400X, bar = 250 μm. F: PC fast setting at 30 days. Dispersed granular structure over a connective congestive tissue was observed. H&E stain. Magnification 400 X, bar = 250 μm. G: MTA at 30 days. Thick granular crystal deposit was discontinued on connective tissues. Magnification 400 X, bar = 250 μm.

setting) and Mineral Trioxide Aggregated (MTA) as experimental groups, and Calcium Hydroxide Paste as positive control group; after their implantation into silicone tubes in rat subcutaneous connective tissues to compared biocompatibility and mineral tissue repair; at short periods of time (24, 72 hours and 7, 15 and 30 days).

Materials and Methods

Animals

Twenty-five male Wistar rats weighing 250 g ± 100 g were obtained from the Animal Research Center (Medical School, Tucumán National University, Argentina). Animals were housed in pairs, in a specific pathogen-free environment, with a temperature of 22.4°C to 23.8°C, relative humidity of 45% to 62%, and a 12-hour light-dark cycle. A standard commercial diet and tap water were available *ad libitum.* The protocol was approved by the local Ethical Committee for Animal Research (Tucumán National University and CONICET). All procedures were developed in accordance with the Guide for the Care and Use of Laboratory Animals 8th edition.

Materials

The materials were divided into a) Control Groups: Silicone: the response of the side wall of silicone tubes acting as a non-toxic, inert material; Calcium Hydroxide: Calen (SS White Dental Articles, Rio de Janeiro, Brazil), as positive control; and b) Experimental Groups: Mineral trioxide aggregate (MTA). ProRoot (Dentsply Tulsa Dental, Tulsa, OK, USA). Portland cement with normal setting (PCn).Cemento Portland with filler calcáreo. (LOMA NEGRA, Buenos Aires, Argentina). Portland cement fast setting (PCf). Practik (KLAUKOL, San Justo, Argentina).

They were prepared according to the manufacturer´s instructions. MTA and PC (fast and normal setting) were prepared in the same way. Three parts of powder and one part of sterile water were mixed until a creamy consistency (30") was obtained.

Surgery

The skin of the animals was shaved and disinfected. An antero-posterior incision was made, creating four pockets. The sterilized silicone tubes (10 mm x 1 mm) filled with the assay materials: Calen, MTA, PCn and PCf, were implanted into each subcutaneous tissue pocket, perpendicular to the incision. The study period was short-term testing and the animals were euthanized by overdose of anesthetic solution (five animals per period) at 24, 72 hours, 7, 15 and 30 days. The tubes and surrounding tissues were removed and fixed in 10% formaldehyde buffer solution, pH 7.

Histological Procedures

Skin samples with implanted tubes and surrounding tissues were included in paraffin blocks, cut into 4-6 μm thick sections. They were stained with Hematoxylin-Eosin (H&E) and Von Kossa. Observations were made with a light microscope at 4X, 10X, 40X, 60X and 100X, and polarized light microscope studies. A single pathologist evaluated all tissues. Subsequently, another pathologist (certified by Argentine Health Ministry License Nº 31455) performed an independent review to verify microscopic observations. The results reported reflect the mutually-agreed-upon diagnoses by both pathologists

The histological parameters were necrosis, acute (polymorphous neutrophils) and chronic (lymphocytes-macrophages) inflammation, abscess, granulation and fibrous tissues, giant foreign body reaction and mineral precipitation, to obtain qualitative scores at the tube end. Grade A. Mild or no inflammation: no local inflammatory reaction appeared either on tissue exposed to experimental material or along the tube. Grade B. Moderate inflammation: Some inflammatory cells, lymphocytes, plasma cells, macrophages, and occasionally foreign body cells at

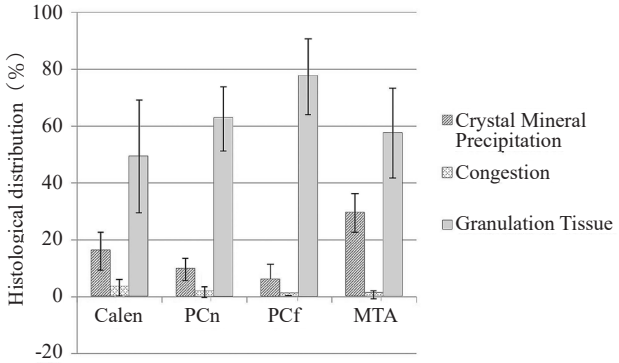

Figure 2. At 30 days shows percentage distribution of crystal precipitation, congestion and granulation tissue compartments from experimental endodontic sealers.

the tube end (analysis area) as well as fibrous tissue along the tube were found. Grade C. Severe inflammation: Necrosis and polymorph neutrophil leukocyte (PMN) collection and lymphocytes, plasma cells, macrophages, fibrous tissue and occasionally foreign body giant cells were observed. The international standard ISO 7405 1984 suggested the following interpretation to establish an accepted or rejected material. A: No or mild reaction at 2 weeks and 12 weeks would be considered acceptable. B: Moderate reaction at 2 and 12 weeks would not be acceptable; moderate reaction at 2 weeks decreasing to 12 weeks would be acceptable. C: severe reaction at any period would not be acceptable.

In addition, morphometric quantitative studies were performed at 30 implantation days in all experimental groups. Three microphotographs at 400X magnification were taken of each sample from the groups[23] at the end of the tube (analysed area) with an Olympus SC50 camera adapted to Olympus BX 43 microscope using CellSens Standard 1.17 Olympus Soft 2009-2017 (Olympus, Tokyo, Japan). The photos were evaluated with an Image Pro Plus analysis system (Version 4.5.0.29 for Windows 1998/NT/2000, Media Cybernetics, Silver Spring, MD, USA). The parameters measured were congestion, granulation tissue and mineral precipitation, expressed as percentages. Foreign body giant cells were counted by field.

Statistical Analysis

Qualitative scores were analysed with Fisher´s Exact Test ($p < 0.05$). The percentage data from the quantitative study were presented as mean and ± standard deviation. analysed using Kruskal Wallis Test ($p < 0.05$).

Results

Histopathological Observations and Qualitative Scores

Negative Control Group: Silicone

At 24 and 72 hours inflammatory cells were observed. At 7 days, thin fibrous connective tissue with few inflammatory cells and fibroblasts was detected. At 30 days thick fibrous connective tissue was found (Fig. 1A). All samples (100%) showed Grade I, no or mild inflammatory reaction (Table 1).

Positive Control Group: Calcium Hydroxide with polyethylene glycol (Calen)

At all time periods analyzed, numerous extensive necrosis areas were observed at the end of tube, where Calen contacts tissues. However, their amount and extension decreased at 30 days. A severe acute inflammatory reaction was observed since 24 h, 72h, 7 days and 15 days. From 15 days to 30 days, granulation tissue was replaced by fibrosis and hyalinization. Calen developed an inflammatory reaction grade B in five samples (22%) and C in 18 samples (78%) (Table 1). Also, at the tube end, numerous crystallized-like structures were deposited in two patterns: 1. cuboid crystals palisade lined and 2. multiple rounded, oval or amorphous formations with discrete, laminated, concentrical calcareous precipitation resembling psammoma bodies, surrounded by giant foreign body cells in 15 samples (Fig. 1B, 1C and 1D).

Table 1. Tissue response distribution in subcutaneous connective tissue by cements expressed as A, B and C scores in percentages. (A) Mild or no reaction: no local inflammatory reaction on tissue exposed to experimental material. Or along the tube, presence of fibrous capsule. (B) Moderate inflammation: A few inflammatory cells, lymphocytes, plasma cells, macrophages, and occasionally foreign body cells at the tube end (analysis area), and fibrous tissue along the tube. (C) Severe inflammation: necrosis and polymorph neutrophil leukocyte (PMN) collection and lymphocytes, plasma cells, macrophages, fibrous tissue, occasionally foreign body giant cells. MTA and PCn setting shows 8% and 4% without inflammation at 30 days.

Material	A	B	C	Total
Silicon	100% (23n)	0% (0n)	0% (0n)	100% (23n)
CaHO	0% (0n)	22% (5n)	78% (18n)	100% (23 n)
PCn	8% (2n)	22% (5n)	69% (16n)	100% (23n)
MTA	4% (1n)	39% (9n)	56% (13n)	100% (23n)
PCfr	0% (0n)	35% (8n)	65% (15 n)	100% (23n)

Experimental Groups

MTA

At 24 and 72 hours, acute inflammatory reaction, congestion, necrosis and hemorrhage were found. At 7 and 15 days a chronic inflammatory reaction, granulation tissue and hyaline deposits were observed. At 30 days, a mature granulation tissue and fibrosis were observed. In addition, crystallized mineral precipitation (Von Kossa positive structure) was distinguished. At the tube end a crystallized precipitation was observed at the beginning of the experiment, although at 30 days the deposit was arranged in randomly scattered layers. The black, brown or white crystals were irregular, globular, rounded or laminated and loosely agglomerated, surrounded by macrophages and foreign body giant cells, at 7, 15 and 30 days. (Fig. 1G). Qualitative response from MTA showed inflammatory reaction B (moderate) in 9 samples (39%) and C (severe) in 13 samples (56%). However, at 30 days, only one sample (4%) was A (mild or no inflammatory reaction) (Table 1).

PCn(normal setting)

At 24 and 72 hours, vascular congestion, hemorrhage and acute inflammatory reaction were observed. Necrosis and abscess were also found. At 7 days, chronic inflammatory cells prevailed and granulation tissue and fibrous connective tissue were deposited. At 15 days granulation tissue and fibrosis with lymphocytic-mononuclear cells infiltrate were observed. A mineral crystal precipitation was found since 24h throughout all study periods. At 15 and 30 days macrophages with phagocytosed particles inside; and foreign body giant cells were observed around those particles (Von Kossa-positive). This disordered amorphous granular mineral precipitation, more scattered than MTA, was deposited on the tube end, surrounded by a hyalinized fibrous for-

mation (Fig. 1E). The qualitative results from PC normal setting showed two samples (8%) with no or mild reaction (A), five samples (22%) with moderate reaction (B) and sixteen samples (69%) with severe reaction (C) (Table 1).

PCf(fast setting)

At 24 and 72 hours, vascular congestion, acute inflammatory reaction with polymorphonuclear neutrophils (PMN), necrosis areas and micro abscesses were observed. At 7 and 15 days chronic inflammatory cells (lymphocytes and macrophages) with granulation and fibrous connective tissue appeared. The mineral crystal precipitation was present since 24hs throughout all study periods. The particles were brown, black or translucent, rounded, larger and less agglomerated compared to MTA (Fig. 1F). At 15 and 30 days, giant foreign body cells and numerous macrophages with phagocytic particles appeared, generating foreign body granuloma in three cases. At 30 days a mineral granular Von Kossa-positive structure was surrounded by hyaline deposit and fibrous tissue. The qualitative results from PC fast setting showed eight samples (35%) with moderate reaction and fifteen samples (65%) with severe reaction (Table 1).

Quantitative Observations

The morphometrical results from experimental groups observed at 30 days after implantation were expressed in bar Fig. 2. The percentages from congestive areas were Calen 3.44% ± 2.87, PCn 1.98% ± 1.88, PCf 0.08% ± 0.18 and MTA 0.7% ± 1.24. No statistical differences were observed ($p = 0.12$). The percentages for granulation tissue area were Calen 49.15% ± 19.76, PCn 62.95 ± 11.42, PCf 77.74 ± 13.31 and MTA 57.74 ± 15.8. There were no statistical differences between groups ($p = 0.50$). Crystallized mineral precipitation percentages values were 16.25% ± 6.9 for Calen, 9.93% ± 3.94 for PCn, 6.32% ± 5.38 for PCf and 29.64% ± 6.96 for MTA. MTA showed statistically significant differences ($p = 0.007$) compared to the other groups. There was counted per 40X field foreign body giant cells (FBGC), by Image Pro Plus analysis system. The media ($\bar{x}$) of FBG Cells found were in Calen 10, PCn, 1, PCf 0,5 and 0 for MTA.

Discussion

In relation to biocompatibility, our results could be considered as *non compatible* according to traditional concepts[21]; or *compatible* according to the present concept[24], based on the ability of a biomaterial, to induce an appropriate and advantageous host response during its clinical usage. Our results showed an A score at 30 days with PCn (8%) and MTA (4%) respectively, in agreement with authors that reported no inflammation. We also found moderate or severe inflammation[10,11,13-16] necrosis and giant cell formation by MTA and PC[25]. Some authors published, similar results at longer time periods[26]. These are not desirable results with respect to adequate tissue response.

Some researchers[11,13-15,25] did not report mineral calcification around subcutaneous implant from MTA and PC materials; while others[11,26-29] described mineral precipitation, although osteo-inductivity was not demonstrated[27]. Although our results showed crystal precipitation, we would not consider it as a calcified bridge, calcified structures, dystrophic calcification or even a biomineralization process. We think that the crystallized structure composed of irregular globular rounded particles, usually surrounded by giant body cells, was not a real new bone calcified bridge. It could be defined as artificial mineralization, which was resorbed in time. It was recently demonstrated that MTA mixed with blood has a negative effect, reducing its hardness, microstructure and compressive strength, and resorbed in time[7]. From *in vitro* studies, calcium hydroxide was found a by-product of PC and MTA hydration, and reacts with phosphates present in synthetic physiological fluids, forming hydroxyapatite[30,31]. However, in *in vivo* studies, there was no crystalline calcium hydroxide formation at early stages; lately it was noted on the surface but not inside the structure. Rounded crystal on the MTA surface and lack of needle-like crystals, characteristic of ettringite mineral formations were observed after contact with blood or synthetic tissue-fluid. Furthermore, in the animal model, no calcium hydroxide formation was observed, and the phases were poorly crystalline[7]. These results agreed with our distinctive disaggregated quartered disordered mineral crystal formation from PC normal and fast setting and MTA. The clinical performance of MTA based on the assumption that the material hydrates and calcium hydroxide were produced after the material had set, developing calcified tissue structures, could not be supported in this study. With Ca HO_2 (Calen), our results with intense (78%) and moderate (22%) inflammatory reaction obtained without mild reaction[17-20] did not agree with a previous popular notion called calcium hydroxide-based sealers as biological sealers[19,32] with a persistent chronic inflammation and foreign body granulomatous reaction observed[17,33-35]. With respect to mineral precipitation, the obtainment of a calcareous palisade at the tube end was appropriate, but not the psammoma body structures found in the granulomatous reaction, associated with either benign or malignant diseases[36,37]. These structures were described here for first time after subcutaneous tissue implantation with OHCa (Calen).

Calcium hydroxide is a strong base[38]. It is slightly soluble in water, releasing Ca and OH ions through ionic dissociation[39]. High pH causes irritation and necrosis, stimulating and forming mineralization, in a process similar to dystrophic calcifications[40]. The dystrophic calcifications observed with Calen in our results could be explained by the slow disaggregation of its components and the development of a foreign body granulomatous reaction. We concluded that OHCa (Calen) was not biocompatible, while highest biocompatibility was observed with PCn (8%) and MTA (4%). MTA developed a thick crystal precipitation compared with PC; however, both mineral formations were distinctive, disaggregated, quartered, irregular, disordered and inconsistent.

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Conflict of Interest

The Authors declares no conflict of interest related to this study.

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